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Connecting the Dots: A New and Complete Salicylic Acid Biosynthesis Pathway

Salicylic acid (SA) or 2-hydroxybenzoic acid, better known as the active ingredient in aspirin, is a phenolic plant hormone that plays an essential role in plant defense against biotrophic and semi-biotrophic pathogens (Fu and Dong, 2013). It is also well known that SA regulates seed germination, stomatal closure, flower development, responses to abiotic stresses, and thermogenesis, etc. (Vlot et al., 2009).

Early studies have demonstrated that SA production during pathogen infection in plants shares similarity to SA biosynthesis in bacteria (Wildermuth et al., 2001). Some bacteria such as *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* can synthesize SA from chorismate via a two-step isochorismate synthase (ICS) pathway, in which chorismate is converted to isochorismate by ICS and then SA is synthesized from isochorismate by isochorismate pyruvate lyase (IPL) (Figure 1A). In *Arabidopsis*, there are two ICS homologs, ICS1 and ICS2. ICS1 is also called SID2 or EDS16. Both *sid2* and *eds16* mutants show a reduced level of SA accumulation in response to pathogen infection. ICS1 is responsible for 90% of pathogen-induced SA production, whereas the second homolog, ICS2, plays a very minor role. ICS catalyzes only the conversion of chorismate to isochorismate, and in bacteria, the IPL enzyme is responsible for converting isochorismate to SA. For this reason, scientists have long searched for an IPL homolog in plants. However, despite many years of intensive research, this putative plant IPL has not been identified (Figure 1A). For close to 20 years, ever since the discovery of ICS1, how SA is synthesized from isochorismate in plants has remained a mystery.

An important milestone in SA biosynthesis was the discovery of PBS3 (avrPphB Susceptible 3) (Warren et al., 1999). PBS3 was identified through genetic screening for *Arabidopsis* mutants compromised in defenses that are mediated by a group of resistance proteins. Further studies demonstrated that *pbs3* mutants show reduced accumulation of SA and SA metabolites. PBS3 is a member of the GH3 family of acyl adenylase enzymes, which conjugate amino acids to an acyl substrate (Figure 1B) (Nobuta et al., 2007). One of the most cited members of this group of enzymes is JAR1, which is responsible for the conjugation of isoleucine to jasmonic acid (JA), producing its active form, isoleucine-JA. The next logical step was to test whether PBS3 could conjugate amino acids to SA. In subsequent experiments, it was discovered that SA is a poor substrate for PBS3. Surprisingly however, PBS3 was found to conjugate amino acids to 4-substituted benzoates (Okrent et al., 2009). The exact function of PBS3 remained another piece of the puzzle in SA biosynthesis at that time. Ten years after the report identifying PBS3, another player *EPS1* (*Enhanced Pseudomonas Susceptibility 1*) was revealed. *EPS1* encodes a BAHD acyl transferase-like protein and plays an important role in SA biosynthesis (Figure 1B) (Zheng et al., 2009), but how EPS1 contributes to pathogen-induced SA biosynthesis was not well understood.

SA inhibits PBS3 enzymatic activity *in vitro* (Okrent et al., 2009), suggesting that SA is a downstream product of PBS3, and SA represses PBS3 activity by feedback inhibition. Therefore, it is more likely that PBS3 conjugates amino acids to isochorismate than to SA. Chen et al. (2009) in a bold prediction, hypothesized that SA is produced from isochorismate by PBS3 followed by EPS1, but exactly how this process is achieved was not clear. Recently, two independent groups took innovative approaches and solved this long-standing mystery. First, both groups generated *Arabidopsis* lines with increased levels of SA in order to amplify their SA signaling (Rekhter et al., 2019; Torrens-Spence et al., 2019). Then they crossed these lines with *pbs3* mutants to generate the same lines in a *pbs3* mutant background. Using high-resolution mass spectrometry, these lines became a powerful tool in dissecting the influence of PBS3 on SA-related metabolites. By comparing metabolomic datasets, they found there were significantly higher isochorismate levels and significantly reduced isochorismate-9-glutamate levels in *pbs3* mutants (Rekhter et al., 2019; Torrens-Spence et al., 2019). This supports the function of PBS3 in conjugating glutamate to isochorismate to produce isochorismate-9-glutamate. To test this hypothesis, both groups had to use purified recombinant ICS1 protein to produce isochorismate from chorismite because isochorismate is not commercially available. Next, both groups incubated isochorismate with L-glutamate and purified PBS3 protein (Rekhter et al., 2019; Torrens-Spence et al., 2019). Isochorismate-9-glutamate was detected as the main product. These experiments prove that PBS3 functions as an isochorismoyl-glutamate synthase conjugating L-glutamate to isochorismate.

How exactly is SA produced from isochorismate-9-glutamate? These two independent groups both demonstrated that SA can be produced from isochorismate-9-glutamate by spontaneous decay (Torrens-Spence et al., 2019). This discovery itself is significant because this would be the first example of a phytohormone that utilizes spontaneous decay for biosynthesis. If SA can be produced from isochorismate-9-glutamate by spontaneous decay, then why do we still need EPS1 for SA biosynthesis? It turns out that EPS1 is unique to the Brassicaceae family (Torrens-Spence et al., 2019). Even though SA can be produced from isochorismate-9-glutamate by spontaneous decay without EPS1, this spontaneous decay happens at a low rate in plants of the Brassicaceae family. Unlike conventional BAHD

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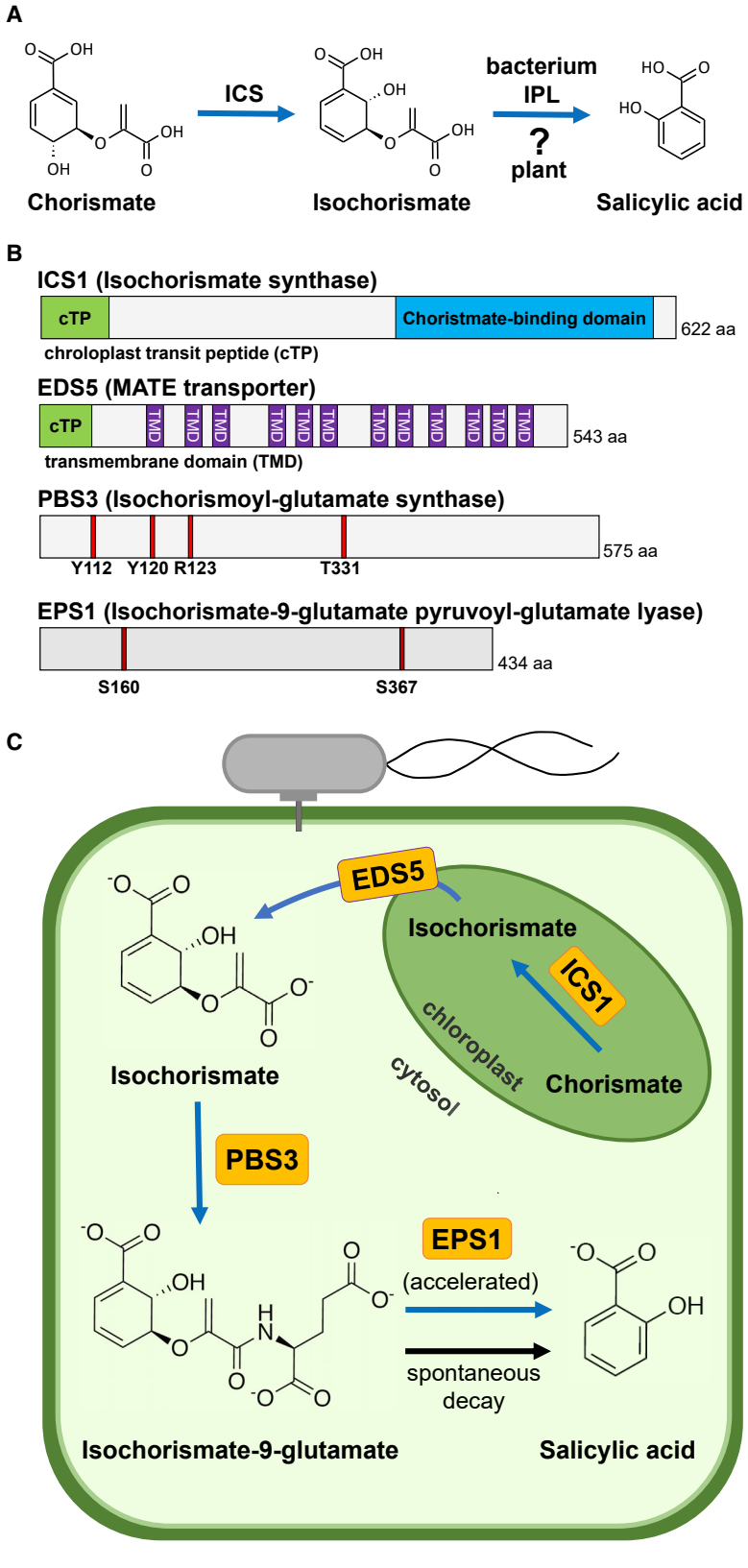

Figure 1. The Complete Pathogen-Induced Salicylic Acid (SA) Biosynthesis Pathway in Plants.

(A) Two-step SA biosynthesis pathway in bacteria. ICS1 converts chorismate to isochorismate, which is subsequently converted to salicylic acid by IPL, an enzyme yet unidentified in plants.

(B) Functional domains and enzymatic activities of key plant proteins involved in pathogen-induced SA biosynthesis. Y120 R123, SA binding sites; Y112 R123 T331, isochorismate binding sites; S160 S367, two serine substitutions at the two conserved catalytic residues important for canonical BAHD acyltransferase.

(C) Model depicting the pathogen-induced SA biosynthesis pathway in plants. In the chloroplast, chorismate is converted by ICS1 to isochorismate, which is transported to the cytosol by EDS5. In the cytosol, PBS3 catalyzes the conjugation of L-glutamate to isochorismate creating isochorismate-9-glutamate, which is then converted to SA by spontaneous decay or at an accelerated rate by EPS1 in Brassicaceae family plants.

acyltransferase, EPS1 has an unusual serine substitution to the highly conserved catalytic histidine in the active site, suggesting that EPS1 may be an unconventional enzyme (Figure 1B) (Torrens-Spence et al., 2019). Further study revealed that in the presence of EPS1, the formation of SA from isochorismate-9-glutamate is four orders of magnitude greater than in the absence of EPS1 (Torrens-Spence et al., 2019). EPS1 functions as an unprecedented isochorismate-9-glutamate pyruvoyl-glutamate lyase (IPGL) to cleave N-pyruvoyl-L-glutamate from isochorismate-9-glutamate, resulting in accelerated production of SA in Brassicaceae family plants (Torrens-Spence et al., 2019). In non-Brassicaceae family plants, based on current knowledge, SA is primarily produced from isochorismate-9-glutamate through spontaneous decay.

Here, we provide a short summary of the new and complete pathogen-induced SA biosynthesis pathway in plants (Figure 1C). Upon pathogen infection, the expression of *ICS1*, *EDS5*, *PBS3*, and *EPS1* will be significantly induced (Wildermuth et al., 2001; Nawrath et al., 2002; Nobuta et al., 2007; Zheng et al., 2009). ICS1 converts chorismate to isochorismate in the chloroplast. EDS5 or SID1, localized in the chloroplast membrane, functions as a MATE transporter (Nawrath et al., 2002), exporting isochorismate to the cytoplasm (Rekhter et al., 2019). PBS3 catalyzes the conjugation of L-glutamate to isochorismate to produce isochorismate-9-glutamate. In Brassicaceae family plants, EPS1 facilitates the production of SA from isochorismate-9-glutamate by functioning as an unprecedented IPGL. In non-Brassicaceae family plants, in the absence of EPS1, SA is produced mainly from isochorismate-9-glutamate by spontaneous decay. To validate their data *in planta*, Torrens-Spence et al. (2019) reconstituted *de novo* SA biosynthesis using *Agrobacterium*-mediated co-expression of SA biosynthesis genes. Co-expression of *SID1-SID2* or *EPS1-SID1-SID2* led to an increased level of isochorismate, which was depleted when *PBS3-SID-SID2* genes were co-expressed, supporting that

SID2 or ICS1 produces isochorismate, SID1 or EDS5 exports isochorismate from the chloroplast to the cytosol (Rekhter et al., 2019), and PBS3 uses isochorismate as a substrate. Co-expression of *SID1-SID2* did not yield a significant level of SA, however, when *SID1-SID2-PBS3* or *SID1-SID2-PBS3-EPS1* were co-expressed, a high level of SA was detected. This indicates that PBS3 is required and SID1-SID2-PBS3 are sufficient for *de novo* SA biosynthesis.

PBS3 and EPS1 are both required for effector-triggered immunity (ETI), whereas ICS1 is not (Warren et al., 1999; Zheng et al., 2009), suggesting that PBS3 and EPS1 have additional functions apart from SA biosynthesis. Future studies on how PBS3 and EPS1 are associated with resistance proteins may help us better understand how PBS3 and EPS1 accomplish their functions in resistance protein-dependent ETI. In addition to ICS1, EDS5, PBS3, and EPS1, pathogen-induced SA production in plants also requires EDS1, PAD4, and NDR1 (Qi et al., 2018). Dissecting the function of EDS1, PAD4, and NDR1 in SA production may help us better understand how SA is produced and/or regulated during plant–pathogen interactions.

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